

CORNEAL IMPLANT AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of United States provisional Patent Application Serial No. 60/391,942 filed June 28, 2002, which is incorporated by reference herein in its entirety.

10 FIELD OF THE INVENTION

 The invention relates to a corneal implant its use for a number of biomedical applications of the cornea, including keratoprosthesis, repair and transplantation. The invention
15 further relates to a method of preparing such a corneal implant.

BACKGROUND OF THE INVENTION

20 The cornea protects the intraocular contents and serves as major optical element of the eye. 75% of the dioptric power of the eye depends on the interface of the cornea and air. Injury, disease or cellular failure can cause opacification of the cornea with subsequent impairment and corneal blindness.
25 Affecting more than 10 million patients worldwide, corneal opacification is often managed by transplantation of human donor tissues. This procedure has a poor success rate in disorders such as autoimmune conditions or chemical injuries. In industrialized countries, the ageing of the population
30 exacerbates this demand, which is compounded by the shelf-life of donated eyes being only a few days. Furthermore, in developing countries where the number of cases of cornea blindness is increasingly problematic, healthy donor tissue is rare. Alternatively, corneal transplants are needed in several

third world countries where donor corneas are not culturally acceptable. Multiple models and devices are necessary to accommodate the various pathological states of the corneas.

5 Polymers and corneal implants

Many attempts have been made to create artificial corneas or keratoprotheses in order to replace donor cornea grafts. Such attempts often failed because of an absence of healing and permanent attachment between the periphery of the synthetic
10 device and the residual rim of host cornea. As a result, tissue necrosis, leakage of aqueous humor, epithelial down-growth, and intraocular infection frequently occurred.

Numerous keratoprotheses have been developed using a variety of polymer materials such as poly-(2-hydroxy ethyl
15 methacrylate) (pHEMA), poly-(methyl methacrylate), polyvinyl alcohol, or poly-(ethyl vinyl alcohol), and in some instances mixed with collagen or hyaluronic acid, (Chirila, 2001 ; US patent 5,458,819 (Chirila et al., issued October 17, 1995); US patent 5,300,116 (Chirila et al., issued April 5, 1994); Hicks
20 et al., 1998a; 1998b; Guisti et al., 1994, Legais et al., 1995 ; 2001; Legeais and Renard, 1998, Robert et al., 2001 ; Trinskaus-Randell et al., 1988; 1997 ; Trinskaus-Randell and Nugent, 1998 ; Vijayasekaran et al., 1998 ; 2000 ; Wu et al., 1998; US Patent 5,436,135 by Tayot et al; July 25, 1995). Most
25 keratoprotheses in development consist of a central transparent optical element, surrounded by a porous opaque material as a peripheral rim that allows penetration and proliferation of stromal keratocytes and the subsequent synthesis of collagen within the material. The peripheral rim
30 has been made of different polymers such as polybutylene-polypropylene, and expanded poly(tetrafluoroethylene). US patent 6,005,160 (Hsiue et al; December 21, 1999) describes a heterofunctional membrane for application as an artificial

cornea using polyacrylic acid or polymethacrylic acid, and then bonded with collagen, or HEMA, or with polyethylene oxide. A hetero, bi-functional biomedical surface can also be developed with 2-methacryloyloxyethyl phosphorylcholine, or

5 2-methacryloyloethyl phosphorylcholine. Such a product has good transparency, hydrophilicity and high biocompatibility. These devices have been more or less successful (e.g., remain in place for 6 months). However, the skirt of most of them have low tensile strength leading to suturability problems,
10 inflammatory reaction, and subsequent extrusion of the keratoprosthesis. A keratoprosthesis made of a transparent pHEMA core is undergoing clinical trials (Chirila et al., 2001).

Other corneal implants have been designed to correct the
15 cornea curvature by inserting an intrastromal implant using polymers in a form of microporous hydrogel material. However, extrusion is still a major issue, as it is undesirable because it tends to cause clinical complications and product failure. For example, a polymerized PEG (by gamma radiation) hydrogel
20 has been designed to be injectable into the stroma (US patent 6,102,946 by Nigam A, issued August 15, 2000). US patent 5,994,133 (Meijs et al; November 30, 1999) reports a corneal implant made with macromonomer of perfluoropolyether. US
25 patent 4,702,244 (Mazzocco; October 27, 1987) reports a polyurethane/collagen hydrogel compound for an intraocular artificial lens. US patent application 20010018612 (Carson DR, published August 30, 2001) describes an introcular lens for long term implantation in cornea composed of two hydrogel
30 materials made of a copolymer of N-vinyl-pyrrolidone and 2-phenylethyl methacrylate and the second polymer is based on glyceryl methacrylate.

Biological and cell-seeded materials

Human amniotic membrane can be used as replacement for full-thickness corneal defects. Although, corneal architecture is normally restored at long term with a layering of the epithelium and endothelium, with pigmentation and vascularization present in the deep layers of the cornea, amniotic membranes are susceptible to infectious contamination and transmission. Type IV collagen from placenta has been proposed to replace amniotic membranes (US patent 5,436,135 (Tayot et al; July 25, 1995)). US patent 5,114,627 (Civerchia; May 19, 1992) describes a collagen hydrogel for promoting epithelial cell growth.

Collagen film or hydrogels can be used as cornea dressing or contact lens (Miyata et al., 1992). Most collagen materials have been chemically crosslinked to increase their resistance to biodegradation.

Alternatively, attempts have been made to reconstruct corneas *in vitro* from cell lines. Individual human corneal epithelial layers have been successfully maintained in culture as a stratified epithelium (Kahn et al., 1993; Araki-Sasaki et al., 1995). Successful reconstructions of corneas comprising the 3 main layers have also been reported using animal cells (Minami et al., 1993; Zieske et al., 1994), and more recently, a whole human cornea equivalent was reconstituted with biological polymers and cells that mimics the physiology of the human cornea and surrounding tissue (Griffith et al., 1999; Griffith et al., published international application PCT/CA99/00057 [published July 29, 1999 as WO 99/37752])). PCT/CA99/00057 relates to reconstruction of *in vitro* cell-based models for use as animal alternatives in irritancy, toxicity, and drug efficacy testing.

There thus remains a great need for improved materials and systems for use in corneal implants, repair and transplantation.

5 SUMMARY OF THE INVENTION

The invention relates to an improved corneal implant or membrane therefor.

Accordingly, in a first aspect, the invention provides a
10 corneal implant comprising a membrane, the membrane comprising a biological polymer and a polyacrylamide.

In an embodiment, the polyacrylamide is a poly(N-alkylacrylamide), such as poly(N-isopropylacrylamide).

In an embodiment, the biological polymer is selected from
15 the group consisting of collagen, fibrin-fibrinogen, gelatin, glycoproteins, peptides, glycosaminoglycans, elastin and mixtures thereof. In embodiments, the peptide is selected from the group consisting of adhesive peptides, growth factors, cytokines and chemokines. The collagen may be selected from
20 the group consisting of telocollagen and atelocollagen, including type I collagen. The collagen may be selected from recombinant collagen and collagen from a natural source. The collagen may be from an animal, e.g. a mammal, such as human, rat (e.g. from rat tail tendon), cow or pig.

25 In an embodiment, the biological polymer and polyacrylamide are present in a ratio of about 0.2:1.0 (w/w) to about 1.0:0.2 (w/w), in a further embodiment in a ratio of about 0.3:1.0 (w/w) biological polymer:polyacrylamide.

The membrane may further comprises chemical crosslinks,
30 e.g. obtained by crosslinking with a carbodiimide crosslinking agent such as 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), or with the combination of a carbodiimide such as EDC

and N-hydroxysuccinimide (NHS), for example using a previously reported method (Olde Damink et al., 1996).

In an embodiment, the cross-linked membrane comprises a characteristic selected from the group consisting of an elastic modulus less than about 10 MPa, a tensile strength at break of less than about 6 MPa, an elongation at break of less than about 20% and a tensile energy to break of less than about 0.5 mJ. In an embodiment, the membrane has a thickness of about 20 μ m to about 400 μ m, in a further embodiment about 50 μ m to about 100 μ m.

In an embodiment, the membrane comprises a characteristic selected from the group consisting of an elastic modulus less than about 10 MPa, a tensile strength at break of less than about 6 MPa, an elongation at break of less than about 80% and a tensile energy to break of less than about 2 mJ.

In embodiments, the implant may comprise a plurality of the above-mentioned membranes. In a further embodiment, the plurality of the above-mentioned membranes collectively comprise a characteristic selected from the group consisting of an elastic modulus less than about 10 MPa, a tensile strength at break of less than about 3 MPa, an elongation at break of less than about 60% and a tensile energy to break of less than about 2 mJ.

The invention further relates to a use of the implant for corneal repair or transplantation.

The invention further relates to a method for treating a corneal injury or defect of a subject, comprising applying to a cornea of a subject the above-mentioned implant. Such a subject may be a mammal, such as a human.

The invention also relates to a commercial package comprising the above-mentioned implant, together with instructions for its use as a corneal implant.

The invention further relates to a method for preparing the above-mentioned corneal implant, the method comprising: (a) providing a polymer mixture solution comprising a biological polymer and a polyacrylamide (e.g. a solution obtained by
5 combining a biological polymer and a polyacrylamide); (b) transferring the solution onto a drying surface; (c) allowing the solution to dry to obtain a membrane for use in the corneal implant. In an embodiment, the biological polymer is collagen.

The method may utilize collagen provided as a solution in
10 0.02N acetic acid in water, having a collagen concentration of about 1.0-6.0 mg/ml, in a further embodiment 3.0-3.5 mg/ml. The method may utilize a poly(N-isopropylacrylamide) which may be provided as a solution of about 2-10% in water, in a further embodiment 4% in water. These solutions may be combined in a
15 1:1 ratio.

The drying surface may be a plastic dish (e.g. a non-treated culture dish) or a mold (e.g. a lens or cornea mold). In an embodiment, the method further comprises a crosslinking step using crosslinking agents such as EDC and/or NHS.

20 In the case where the implant comprises a plurality of membranes, the method further comprises layering together a plurality of the membranes so obtained. In an embodiment, the method further comprises crosslinking the membranes to each other, using for example the agents and methods described
25 herein.

The invention further relates to a use of a membrane comprising a biological polymer and a polyacrylamide as a corneal implant.

The invention further relates to a use of a plurality of
30 membranes as a corneal implant, wherein at least one of said membranes comprises a biological polymer and a polyacrylamide. In an embodiment, the plurality of membranes comprises inter-membrane chemical crosslinks. In an embodiment, the crosslinks

are obtained by crosslinking with a crosslinking agent selected from the group consisting of (a) a carbodiimide crosslinking agent; (b) an N-hydroxysuccinimide; and (c) both (a) and (b). In an embodiment, the carbodiimide crosslinking agent is 1-(3-
5 dimethylaminopropyl)-3-ethyl carbodiimide.

The invention further relates to a commercial package comprising a membrane, the membrane comprising a biological polymer and a polyacrylamide, together with instructions for its use as a corneal implant.

10 The invention further provides a method for treating a condition characterized by a corneal defect, comprising applying the implant of the invention to a cornea of a subject. In an embodiment, the subject is a mammal, in a further embodiment, a human.

15 The invention also provides a commercial package comprising the implant of the invention together with instructions for treating a condition characterized by a corneal defect.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Morphological aspect of the collagen-pNIPAAm specimens. In A, the collagen-PNIPAAm exhibits some collagen fibrils in solution prior to the drying process. The wet
25 mixture appeared similar to the mixture obtained following the drying process. In B, histological sections of pNIPAAm-collagen membranes are presented. If a 10-20% size reduction due to the formaldehyde fixation is considered, membrane thickness is about 300-400µm. Collagen fibrils can be seen
30 relatively oriented in the plane of the membrane. In C, pNIPAAm has been mixed with fibrin polymer, the resulting gel consisting of a network of filamentous structures. Bar = 100µm.

Figure 2: Quantification of the growth of endothelial cells seeded in plastic dish in the presence of 10% fetal calf serum and endothelial cell growth supplement for 4 days. During this period, solutions of pNIPAAm were added at different dilutions in culture medium. Cell growth was not impaired by the presence of the polymer in cultures. Statistic analysis did not show any significant difference between these concentration conditions.

Figure 3: Cornea observation after collagen-pNIPAAm membrane implantation in a cornea defect in rabbit eye. At day one, the membrane is in place well secured and transparent (A). The fluorescence shows the wound site (B). By day six, the defect is still clear (C) and fluorescence staining is minimal (D) that correspond to the presence of a reepithelialisation of the implant.

Figure 4: Histological section of the collagen-pNIPAAm (A) and amniotic membrane (B) implant in rabbit cornea after 12 days of implantation. The collagen-pNIPAAm implant (arrows) is present within the stromal regeneration and the implant is uniformly covered with host epithelial cells. The stromal reaction is well oriented as in normal stroma with stromal cells between collagen fibers. No inflammatory or immune cells can be seen in the nearby of the implant. In B, the amniotic membrane is likely to be biodegraded and a epithelial hyperplasia can be observed in the periphery of the cornea defect. In C, the microscopic image of the collagen-pNIPAAm implant shows that the polymeric membrane (pm) is completely innervated 3 months after surgery. Nerves (arrowheads) are observed extending into the polymer. Bar = 250µm.

DETAILED DESCRIPTION OF THE INVENTION

The invention is in the field of ophthalmology. In embodiments, the invention relates to a membrane which serves
5 as the basis of a substitute for corneal implant or graft (i.e., penetrating keratoplasty), keratoprosthesis and to a replacement of amniotic membrane used as corneal implant and support for cultured epithelial and precursor cells. In
10 embodiments, the substitute, i.e. the membrane or corneal implant of the invention, can be transplanted onto the cornea to assist cornea wound healing after eye surgery, cornea injury and disorders, or excimer laser surgery.

In an aspect, the invention relates to a corneal implant or keratoprosthesis comprising a membrane of the
15 invention, which is produced by the combination of biological polymer, such as collagen, and a polyacrylamide, e.g. a polyalkylacrylamide such as poly(N-isopropylacrylamide) (pNIPAAm). The use of such synthetic polymers is advantageous as it provides good transparency and strength to the membrane,
20 contributing to its use as a corneal implant. Prior to the studies described herein, pNIPAAm and its derivatives have not been used in any cornea or ocular device. In an embodiment, the biological polymer is collagen, in a further embodiment, type I telocollagen, a soluble form of non-pepsinized collagen
25 that may for example be purified from rat tail tendon (RTT). Other sources of collagen, including mammalian sources, such as bovine, porcine, and human telocollagen or atelocollagen (pepsin or pronase digested telocollagen) may also be used. Collagen may also be from a recombinant source. In the
30 examples described below, the synthetic polymer pNIPAAm homopolymer has been used.

"Corneal implant" as used herein refers to any material or device which may be applied to or comes in contact

with the cornea of a subject. In an embodiment the subject is a mammal, in a further embodiment, a human. Such an implant may be used for repair or replacement of a cornea or a portion thereof, or as a cornea wound dressing, due to defects for
5 example caused by corneal injury or disease. In a preferred embodiment, the implant is substantially transparent.

pNIPAAm-based materials

The pNIPAAm family of polymers is one of the few synthetic
10 materials which support cell in-growth and growth of polymer encapsulated cells (Vernon et al 1999; Stille et al 1999). pNIPAAm has been extensively studied *in vitro* for cell culture use that demonstrates its non cytotoxicity (Takezawa et al 1990). Takezawa et al. (1992) have combined collagen with
15 pNIPAAm as cell culture substrate in order to form spheroids, producing only a thin (2 μ m) layer of collagen-pNIPAAm. Moreover, US patent 6,030,634 (Wu et al.; February 29, 2000) describes the combination of pNIPAAm with gelatin (denaturated collagen) which results in a polymer gel having the properties
20 of an interpenetrating polymer network structure with a shrinking temperature of 35°C, having surgical application for the repair of damaged tissue, but not replacement or substitution. Further, corneal applications are not contemplated nor mentioned. An improved shrinking rate for a
25 drug delivery system using such network structure is also described. Furthermore, activated pNIPAAm has also been conjugated to protein A, various enzymes, biotin, phospholipids, RGD peptide sequences, and other interactive molecules such as monoclonal antibodies (as described in U.S.
30 patent 4,780,409; October 25, 1988; Monji and Hoffman, 1987).

The polymer gel compositions of the present invention comprises a matrix, such as an interpenetrating pNIPAAm-collagen hydrogel network which leads to strengthen the final

collagen-based product while keeping its transparency. In
embodiments, the membrane of the invention may be made with
different ratios of collagen to pNIPAAm. In an embodiment, the
collagen to pNIPAAm ratio is in the range of about 0.2:1.0 to
5 1.0:0.2 (w/w). In an embodiment, the collagen to pNIPAAm ratio
is about 0.3:1.0 w/w, which is the preferred ratio for corneal
implant design.

Collagen can be replaced or mixed with other biological
polymers, including proteins such as gelatin, fibrin-
10 fibrinogen, glycosaminoglycans, elastin, and glycoproteins or
peptides such as adhesive peptide sequences, cytokines,
chemokines or growth factors.

The membranes can be layered together, in a further
embodiment laminated together, to form a composite material in
15 which thickness can be a controlled parameter. In addition, a
multilayered membrane may result in the guidance of the stroma
regeneration within the implant. As such, in an embodiment, a
corneal implant of the invention may comprise a plurality of
the membranes of the invention. The layered membranes in such
20 a structure may be heterogeneous, i.e. having different
polymeric compositions and ratios of components. In addition,
a mixture of crosslinked and non-crosslinked membranes may be
used. The membranes in such a layered structure may be
crosslinked to one another using for example the crosslinking
25 agents and methods described herein.

A variety of agents or compounds (crosslinking,
plasticizer, etc.) can be introduced during manufacturing of
the membrane before and/or after the formation of the membrane.
Depending on the step in which the agent or compound is added,
30 the physicochemical and biological properties of the membrane
may vary. In embodiments, the membrane of the invention may be
crosslinked with different agents. In an embodiment, EDC and
NHS are the crosslinking agents.

pNIPAAm can be modified physically to provide different biophysical and biological properties for different ophthalmic applications (e.g., wound dressings) and mixed with collagen. In a further embodiment, collagen membranes can be crosslinked
5 chemically either during the procedure or afterwards.

In an embodiment, the membrane is transparent and its thickness range is between about 20 μ m and about 400 μ m, depending upon hydration. In a further embodiment, the thickness of the membrane is in the range of about 50 μ m to
10 about 100 μ m. Different thicknesses can be produced, as well as different front and back curvature for cornea replacement.

In an embodiment, the membrane comprises an elastic modulus of less than about 10 MPa, a tensile strength at break of less than 6 MPa, an elongation at break of less than 80% and
15 a tensile energy to break of less than 2 mJ.

In an embodiment, the membrane of the invention is strong enough to support suturing stresses. Its physical properties such as strength and elasticity can be modified by crosslinking (e.g. with carbodiimide-type crosslinking agents such as EDC),
20 but other crosslinking agents (e.g., NHS) and plasticizers such as glycerol can be used. The physical properties of the membrane may be modified for example as a function of rehydration, or via the presence of lipids and/or proteins. Physical modification of pNIPAAm can lead to different
25 properties that may enhance biological and biophysical characteristics for corneal implants or membrane substitutes.

The membrane of the invention provides a device which remains robust and optically clear in the eye for extended periods of time.

30 In embodiments, there is cell ingrowth into the polymeric membrane, and epithelialization over the membrane with no hyperplasia. The membrane may induce the deposition of organized extracellular matrix proteins resembling the cornea

stroma with close reorganisation of the newly deposited collagen and stromal cells.

The membrane of the invention may further comprise/have associated with it various compounds e.g. drugs, biological materials (e.g. peptides/proteins, lipids, etc.), crosslinkers, plasticizers, cytokines, etc. to fulfill or further contribute to an aspect of the desired functionality of the corneal implant in any particular situation. Such agents or compounds may be introduced during the making of the membranes or after their formation.

The invention further relates to a method of preparing a corneal implant of the invention, comprising combining a biological polymer and a polyacrylamide (or providing such a mixture), and allowing the mixture to dry to form a membrane for use as a corneal implant. In embodiments, the biological polymer is collagen provided as a collagen solution of about 1-6 mg/ml, in a further embodiment, about 3.0-3.5 mg/ml (e.g. in 0.2N acetic acid in water), and the polyacrylamide is pNIPAAm provided in a solution of about 2-10%, in a further embodiment, about 4% in water. In an embodiment, the solution of the biological polymer and the polyacrylamide may be combined in a ratio of about 1:1. The polymer mixture may be poured into a suitable dish (e.g. a plastic culture [e.g. non-treated] dish) or mold (e.g. a lens or cornea mold) for drying. Drying may be allowed to proceed until for example a constant weight (e.g. about 1-10% water residue, in a further embodiment about 7% water residue) is reached. Drying may be performed under sterile conditions, under a laminar flow hood. Drying may be performed at room temperature (15-22°C), for example for a period of about 2-4 days. The preparation may further involve a crosslinking step, using for example a crosslinking agent such as EDC and/or NHS. The resulting membrane is typically

rehydrated with a suitable solution prior to its use for cornea implantation.

The invention further relates to the use of a membrane of the invention as a corneal implant, e.g. for application to the cornea for repair or replacement of damaged or otherwise
5 inadequate cornea, thus for the treatment of a corneal defect, disorder, injury or disease.

The invention further relates to a method of treating a condition characterized by a corneal defect (e.g. corneal
10 disorder, injury or disease), said method comprising administering to a subject in need thereof a corneal implant of the invention.

The invention further relates to a commercial package comprising a membrane of the invention together with
15 instructions for its use as a corneal implant, e.g. for application to the cornea for repair or replacement of damaged or otherwise inadequate cornea, thus for the treatment of a corneal defect, disorder, injury or disease.

Although various embodiments of the invention are
20 disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result
25 in substantially the same way. Numeric ranges are inclusive of the numbers defining the range. In the claims, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to". The following examples are illustrative of various aspects of the
30 invention, and do not limit the broad aspects of the invention as disclosed herein.

Throughout this application, various references are referred to describe more fully the state of the art to which

this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

5 EXAMPLES

Example 1: Collagen-pNIPAAm membranes

A sterile RTT collagen solution of 3.0-3.5 mg/ml (w/v) in
10 acetic acid (0.02N in water) is made, and kept at 4°C. A 4%
(w/v) solution of pNIPAAm homopolymer is made in water (ddH₂O)
and sterilized by autoclaving or by filtering (0.24µm). The 4%
solution of pNIPAAm is diluted to a 1% solution with sterile
ddH₂O. The 0.30-0.35 % solution of collagen and the 1%
15 solution of pNIPAAm are mixed (1:1 vol/vol) in a sterile test
tube at 4°C by pumping with a pipette until well dispersed.
Cold mixing will avoid any premature gelification or
fibrillogenesis of the collagen during this procedure.
Collagen-pNIPAAm is then poured into a plastic dish (non-
20 treated culture dish) or a mold (e.g., lens or cornea mold) and
left air-drying under sterile conditions in a laminar flow hood
for at least 2-3 days at room temperature. After drying to
constant weight (~7 % water residue) at room temperature, under
laminar flow at relatively constant humidity, the formed
25 membrane is removed from the mold after soaking for a short
period (hours) in a sterile buffered solution (e.g., Hank's
balanced salt solution or HBSS) at room temperature. The
sterile membrane is ready to be used for cornea implantation or
for a transplantable membrane support for epithelial cells.
30 Conversely, RTT collagen solution that is not mixed with
pNIPAAm as reported above, and only diluted in water or buffer
at 1:1 ratio, fails to become a transplantable and suturable
collagen membranes after drying.

Example 2: Fibrin-pNIPAAm membranes

A sterile fibrinogen (fraction I; type I-S from bovine
5 plasma) solution (3mg/ml in HBSS) is mixed with a 1% pNIPAAm
solution as reported in Example 1. During mixing, thrombin
(50U/ml purchased from Parke Davis) is added at a final
concentration of 0.03:1 v/v (thrombin:fibrin), and incubated
10 for 10-15 min at 37°C to allow gelification (hydrogel) in a
culture well. This procedure strengthens the fibrin gel,
probably by aiding the development of a filamentous network as
shown on Figure 1 C.

Example 3: Crosslinked membranes

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A sterile RTT collagen viscous solution and a 1% (w/v) solution
of pNIPAAm are made as described in Example 1. The 0.30-0.35 %
solution of collagen and the 1% solution of pNIPAAm are mixed
(1:1 vol/vol) with a 10% stock solution of 1 -(3 -
20 dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC)
in water to give a final EDC concentration of 0.5; 0.1; or
0.05% (w/v) in a sterile test tube at 40°C by pumping with a
pipette until well dispersed. The collagen-pNIPAAm-EDC mixture
is then poured into a plastic dish (non-treated culture dish)
25 or a mold (e.g., lens or cornea molds) and left air-drying
under sterile conditions in a laminar flow hood for at least 2-
3 days at room temperature. After drying to constant weight
(~7 % water residue), an interpenetrating network of pNIPAAm in
cross-linked collagen is formed. Each membrane is removed from
30 the mold after soaking in a sterile buffered solution (e.g.,
HBSS) at room temperature. Among the three different
concentrations of EDC tested, the one with the lowest
concentration gave the most reliable procedure to toughen the

membrane. The sterile membranes can be soaked in HBSS containing glycine (5% in HBSS) to remove residual unreactive crosslinking agent. Membranes are further rinsed in HBBS (at least 3 times). Thus, they are ready to be used for cornea
5 implantation or for a transplantable membrane support for epithelial cells.

Example 4: Alternative thick membranes

10 Membranes from Examples 1 and 3 are used to form a thicker membrane that are produced by sticking one or several membranes together. For this procedure, hydrated membranes are bound to each other using a solution of 0.5% EDC solution. The resulting membranes of various thicknesses are left to dry,
15 then rinsed as described in Example 1.

As an example of procedure:

- after rehydration of the membrane, excess water is removed
- one rehydrated membrane is left bonded to the bottom of the
20 dish or mold
- the other membrane to be glued to the first one is released from its dish or mold
- the EDC solution (10 μ l of 10% stock EDC solution per cm^2 of membrane) is poured over the unreleased membrane. EDC plays the
25 role of a glue between the 2 membranes.
- at once, the second membrane (released one) is laid down onto the solution and stuck to the unreleased membrane,
- combined membranes are dried, later rehydrated, and finally rinsed as described in Example 1.

30

Different combinations can be made such as one crosslinked membrane sandwiched between two uncrosslinked membranes, or other alternatives.

Example 5: Incorporation of compounds

A variety of agents or compounds (e.g., crosslinking,
5 plasticizer, drugs, cytokines) can be introduced during the
making of the membranes from examples I to IV. Compounds can
be introduced either during the mixing of both collagen and
pNIPAAm or after the formation of a membrane. The latter can
be dried, thereby, the agents can be introduced during the
10 rehydration process. Otherwise, the agents can be introduced
on the rehydrated membrane. The physicochemical and biological
properties may vary (Table I).

Example 6: Physical properties

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The rehydrated implants from examples 1 and 3 to 5 are
strong enough to support surgical manipulation, suture thread
and needle (Table I). They are relatively flexible. The
presence of proteins and lipids (e.g., Albumax®) is likely to
20 strengthen the membranes. Similar properties are also observed
in the presence of glycerol combined at the time of mixing
collagen and PNIPAAm solutions. The membrane thickness can
vary from 20 to 400 μm after hydration (Figure 1).

TABLE I: Shearing characteristics of the resulting membranes produced in different experimental conditions. Shearing forces are roughly determined by handling the specimen between 2 forceps in opposite directions. The membranes result from the mixture of collagen and PNIPAAm. Collagen can be prepared by making a solution either in acetic acid at pH 4.0 (Coll(ac.ac.)) or in water at pH 3.0 with HCl(Coll (HCl)). A crosslinking agent such as EDC is introduced either during the mixture or after the formation of a membrane. The latter fragilizes the membrane. Glycerol can be used as a plasticizer. Albumax (a lipid rich bovine serum albumin, purchased from GIBCO/BRL) can be added during rehydration with Hank's balanced salt solution (HBSS). Other components can be introduced at different periods to induce different properties.

Collagen (Coll)	Additional components added to the collagen- PNIPAAm mixture	Components added during rehydration (HBSS+)	Results (N=1-2)
Coll (ac. ac.)			strong
Coll (HCl)			strong
Coll (HCl)	0.05% EDC		very strong
	0.1% EDC		strong
	0.5% EDC		more fragile
Coll (HCl)		0.1% EDC	fragile
		0.5% EDC	fragile
Coll (ac. ac.)	0.5% Glycerol		strong
	1% Glycerol		strong
Coll (ac. ac.)		Albumax (2X)	very strong

Example 7: Biomechanical properties

Composite collagen-PNIPAAm membranes from Example 1 were uncrosslinked (1M) or crosslinked by a carbodiimide derivative, the 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (1Mes); other membranes were layered by chemically binding 2 sheets of the collagen-PNIPAAm composite which were uncrosslinked (2M) or crosslinked (2Mes). Chemical binding was performed with the same carbodiimide.

Biomechanical tests were performed on membranes using an Instron Apparatus (Canton, MA, USA). Strips of membranes were placed between the two grips of the apparatus and uniaxial tension was applied. Elongation of the membranes were recorded and analyzed (Table II).

Table II. Biomechanical assessments of uncrosslinked and crosslinked membranes. Means and standard errors of means are presented. These results were from 2 separate experiments of 3 samples each.

	Elastic Modulus MPa	Tensile Strength at break MPa	Extensibility at break % elongation at break	Tensile Energy to break mJ
1M	0.26 \pm 0.06	0.12 \pm 0.03	67.1 \pm 14.7	0.17 \pm 0.03
1Mes	1.9 \pm 0.78	3.27 \pm 1.72	8.34 \pm 4.8	0.19 \pm 0.07
2M	2.73 \pm 0.41	0.73 \pm 0.11	50.1 \pm 4.03	1.01 \pm 0.16
2Mes	6.26 \pm 3.03	1.41 \pm 0.64	8.05 \pm 2.63	0.08 \pm 0.02

Carbodiimide crosslinking of collagen-PNIPAAm improved the strength of the membranes, particularly with the one-layer membrane, which exhibited an associated increased elasticity and toughness. The two-layered membranes were very elastic, probably due to a sliding effect within the 2 membranes. Although the strength of the two-layered membranes

was weaker than the one-layer membrane, the overall toughness was sustained. The biomechanical properties of the membranes are similar to those reported for the human cornea (Zheng et al., 2001; Wang et al., 2001).

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Example 8: *In vitro* cell growth

Human stromal cells or keratocytes were seeded at 5×10^5 cells per cm^2 onto the collagen-pNIPAAm membranes from Example 1. They were then grown in the presence of culture medium and serum for 7 days. Stromal cells form a cell layer on the surface of the membrane as seen on histological section (see Figure 1).

In other experiments, pNIPAAm was dissolved in the culture medium. Human endothelial cells were grown in the presence of the polymer for 5 days at different concentrations (0; 0.06; 0.12; 0.25 and 1.0 mg/ml). Endothelial cell numbers were determined by spectrofluorometry after staining cells with a DNA-specific dye (Hoechst 33342). Cell growth was not impaired by increasing concentration of pNIPAAm (no significant difference by ANOVA test) (see Figure 2).

Example 9: *In Vivo* Biocompatibility

Collagen-pNIPAAm membranes from Example 1 were implanted into the corneas of 15 rabbits. Controls included sham operations and use of human amniotic membrane. Figures 3A&B show rabbit corneas at 3 days after surgery and fluorescein staining of the wound, respectively. By 3 days post-operative, re-epithelialization had occurred and fluorescein staining was minimal with the collagen implants. Conversely, 3 out of 9 rabbits with amniotic implants did not epithelialize by 3 days (Figures 3C&D). After 6 days a complete epithelialization was

observed by direct observation and there was no fluorescein staining. This was comparable to observations made on corneas covered with amniotic membranes, which are now being used clinically to treat corneal epithelial damage due to disease or injury. Biopsies were taken at 12 days and show a complete epithelialization of the surface of the collagen-pNIPAAm membrane that was in place and non degraded (Figure 4A). Around the implant the formation of an oriented stroma was observed, that is organized like the nearby stroma of the cornea. There was an absence of immune and inflammatory reaction. Furthermore, histological section of the cornea with the amniotic membrane showed a partial degradation of the membrane and a hyperplasia of the epithelial cell layer at the nearby of the cornea pocket (Figure 4B). In addition, regenerating nerve axons were observed at the edges of the polymeric membrane underlying the wound at 11 days post-surgery and nerves had penetrated the periphery of the polymers by 28 days. By 3 months post-surgery, there was nerve re-growth into the polymeric membrane (Figure 4C). Moreover, fine neurites are observed within the polymer-epithelial interface. Nerve regrowth into the membrane and overlying epithelium are consistent with previous observations of cornea nerve regeneration in rabbits (Tervo et al., 1994) and demonstrate the feasibility of transplantable artificial corneas that promote nerve in-growth following surgery. These artificial corneas could address the world donor cornea shortage problem while circumventing the problems resulting from the lack of nerve regeneration found in artificial keratoprosthesis.

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